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*Full Length Research Paper*

# Characterization of the *Antheraea pernyi abnormal wing disc* gene that may contribute to its temperature tolerance

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**It has been known that the *abnormal wing disc* (*awd*) gene encodes a nucleoside diphosphate kinase and is closely related to wing development in *Drosophila melanogaster* and *Bombyx mori*. In the present study, the *awd* gene was isolated and characterized from *Antheraea pernyi*, a well-known wild silkmoth. The isolated cDNA sequence is 666 bp in length with an open reading frame of 462 bp encoding a polypeptide of 153 amino acids, which contains a putative nucleoside diphosphate kinases active site motif and conserved multimer interface. The deduced *A. pernyi awd* protein sequence reveals 75, 82 and 96% identity with its homologue of *Homo sapiens*, *D. melanogaster*, and *B. mori*, respectively. Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis showed that the *awd* gene was transcribed during all four developmental stages (egg, larva, pupa, and moth), and present in all tissues tested (blood, midgut, silk glands, Malpighian tublues, spermaries, ovaries, brain, muscle, fat body and body wall), with the highest abundance in Malpighian tubules. Interestingly, mRNA expression level in pupal fat body was significantly down-regulated after cold shock (4°C) compared with the control (26°C) and significantly up-regulated after heat shock (46°C). The results indicated that the *A. pernyi awd* gene is inducible, and that its expression effect is different after cold stress and heat stress. Consequently, we refer that the product of the *awd* gene may contribute to its temperature tolerance.**

**Key words:** *Antheraea pernyi*, *abnormal wing disc* gene, cloning, expression pattern, temperature stress.

## INTRODUCTION

Nucleoside diphosphate kinases (NDK) are enzymes required for the synthesis of nucleoside triphosphates (NTP). They provide NTPs for nucleic acid synthesis, cytosine triphosphate (CTP) for lipid synthesis, uridine signal transduction and microtubule polymerization. NDK triphosphate (UTP) for polysaccharide synthesis and guanosine triphosphate (GTP) for protein elongation, are proteins of 17 kDa that act via a ping-pong mechanism in which a histidine residue is phosphorylated by transfer of the terminal phosphate group from adenosine triphosphate (ATP). In the presence of magnesium, the phosphoenzyme can transfer its phosphate group to any nucleoside diphosphates (NDP), to produce a NTP. Mammalian NDK is also known as metastasis inhibition factor 23 nm (nonmetastatic gene 23) which is differentially expressed in certain metastatic tumors. NDK isozymes have been sequenced from prokaryotic and eukaryotic sources including many insects (Biggs et al., 1990; Gilles et al., 1991; Drosophila 12 Genomes Consortium, 2007; Ribeiro et al., 2007; Calvo et al., 2009); the sequence showing that it has been highly conserved through evolution.

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**Abbreviations: NDK,** Nucleoside diphosphate kinases; ***awd*,** *abnormal wing disc*; **NTP,** nucleoside triphosphates; **GTP,** guanosine triphosphate; **CTP,** cytosine triphosphate; **UTP,** uridine triphosphate; **ATP,** adenosine triphosphate; **NDP,** nucleoside diphosphates; *btl*, breathless; **EST,** expressed sequence tag; **ORF,** open reading frame; **BLAST,** basic local alignment search tool; **RT-PCR,** reverse transcriptase-polymerase chain reaction; **UTR,** untranslated region.

It has been shown that the *Drosophila awd* (*abnormal wing discs*) protein is a microtubule-associated NDK (Biggs et al., 1990). The product of the *awd* gene of *Drosophila* is 78% identical to the product of the 23 nm gene of mammals. Neuroblasts in *Drosophila* larvae homozygous for a null mutation in the *awd* gene were arrested in metaphase, indicating that microtubule-associated *awd*/NDP kinase plays a critical role in spindle microtubule polymerization. The *awd* gene is normally expressed in some tissues where it is not required (Xu et al., 1996). This NDK activity is necessary for the biological function of the *awd* gene product. Complete loss of function, that is, null, mutations cause lethality after the larval stage. Most larval organs in such null mutant larvae appear to be normal, but the imaginal discs are small and incapable of normal differentiation (Rosengard et al., 1989; Biggs et al., 1990). Loss of *Drosophila melanogaster awd* protein results in dysregulated tracheal cell motility, a phenotype that can be suppressed by reducing the dosage of the chemotactic FGF homolog breathless (*btl*), indicating that *btl* and *awd* are functionally antagonists (Dammai et al., 2003). There may be additional *Drosophila* genes that encode proteins with nucleoside dipthosphate kinase activity (Timmons and Shearn, 2000). The *awd* gene of *Bombyx mori*, a lepidopteran model insect, has also been thought to be closely related to wing development (Zhao et al., 2009).

*Antheraea pernyi* is one of the most well-known wild silkmoths of the family Saturniidae of order Lepidoptera. Its exploitation for sericulture was initiated in China about 2000 years ago (Liu et al., 2010). It is commercially cultivated mainly in China, India, and Korea for silk production. At present, it is also used as a source of insect food (larva, pupa, moth) and for cosmetics. Moreover, with the development of biotechnology, this species has become an excellent natural bioreactor for the production of recombinant proteins due to its large body and pupal-diapause for long-period (Huang et al., 2002). Despite its importance, only about 40 functional genes of *A. pernyi* were cloned and partially studied to date (Liu and Jiang, 2008). In our laboratory, we have constructed a full-length cDNA library for *A. pernyi* pupa and performed the whole cDNA sequencing to identify more functional genes in relation to diapause and tolerance (Li et al., 2009).

In this paper, the *A. pernyi awd* gene was identified during random sequencing of the full-length cDNA library of *A. pernyi* pupa. We compared the *A. pernyi awd* protein to other insects and determined the expression patterns of the *awd* gene at various developmental stages and different tissues. Finally, the effects of cold and heat shocks on the relative mRNA expression pattern are determined.

## MATERIALS AND METHODS

### Insects and tissues dissection

The *A. pernyi* strain *Shenhuang No. 1* was used in this study. Larvae were reared routinely on oak trees (*Quercus liaotungensis*) in the field. Blood, fat body, midgut, silk glands, body wall, Malpighian tubules, spermaries, ovaries, brain and muscle were taken from silkworm larvae at day 10 of the fifth-instar and were dissected, immediately frozen in liquid nitrogen and stored at -80°C. Eggs at day 5, fifth-instar larvae, pupae and moths were also frozen in liquid nitrogen and stored at -80°C for later use.

To examine the effect of temperature stress on the mRNA expression of the *awd* gene, three stress conditions, including 46°C heat shock treatment for 3 h, 26°C control treatment for 3 h and 4°C cold shock treatment for 3 h, were used. These three stress conditions were used according to the treatment conditions previously reported on *B. mori*, a lepidopteran insect model for research (Lee et al., 2005) and on *A. pernyi* (Xia et al., 2009). The temperature for heat shock was changed from 43 - 46°C after preliminary experiment. After treatment, fat body was immediately dissected from female pupae to extract total RNA. Tissue samples from 18 silkworm pupae were pooled randomly to three groups for each treatment.

### Mining of the *A. pernyi awd* gene and sequence analysis

A SMART cDNA library of *A. pernyi* was constructed in our laboratory (Li et al., 2009), and the randomly selected positive clones were sequenced. An expressed sequence tag (EST) encoding the *awd* homologue (GenBank accession no. GH334841) was isolated. So, the cDNA clone was picked and used to complete the full-length cDNA sequence of the *A. pernyi awd* gene (GU073317). DNA sequences generated were assembled and edited to obtain a consensus sequence using Staden Package (Bonfield et al., 1995). DNASTAR software (DNASTAR Inc., Madison, Wisconsin, USA) was used to identify open reading frame (ORF) and deduce amino acid sequence. Predictions of isoelectric point and molecular weight were also carried out by DNASTAR software. Basic Local Alignment Search Tool (BLAST) search was performed at http://www.ncbi.nlm.nih.gov/blast/. The amino acid sequence was submitted to predict protein signal peptide with SignalP server online tools, http://www.cbs.dtu.dk/services/SignalP/. Transmembrane protein topological structure was analyzed with TMHMM server online tools, http://www.cbs.dtu.dk/services/TMHMM/. Conserved domains was predicted at http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi/. Prosite was scanned at http://www.expasy.org/tools/scanprosite/. Subcellular localization was predicted by SubLoc at http://www.bioinfo.tsinghua.edu.cn/SubLoc/. The *in silico* gene expression analysis based on the available EST resources was employed at http://www.ncbi.nlm.nih.gov/Unigen/ESTprofileViewer/.

### Phylogenetic analysis

The amino acid sequences of the *awd* genes from different organisms used for phylogenetic analysis were downloaded from GenBank database. Multiple sequence alignments were carried out using Clustal X software (Thompson et al., 1997). A phylogenetic tree was constructed by MEGA version 4 (Tamura et al., 2007) using Neighbour-Joining (NJ) method (Saitou and Nei, 1987) with bootstrap test of 500 replications.

### Total RNA extraction and first strand cDNA synthesis

Total RNA was extracted from *A. pernyi* samples using RNAsimple Total RNA Extraction Kit (TIANGEN Biotech Co. Ltd., Beijing) according to the manufacturer's instructions. DNase I was used to remove contaminating genomic DNA. The purity and quantity of extracted RNA was quantified by the ratio of $OD_{260}/OD_{280}$ with an

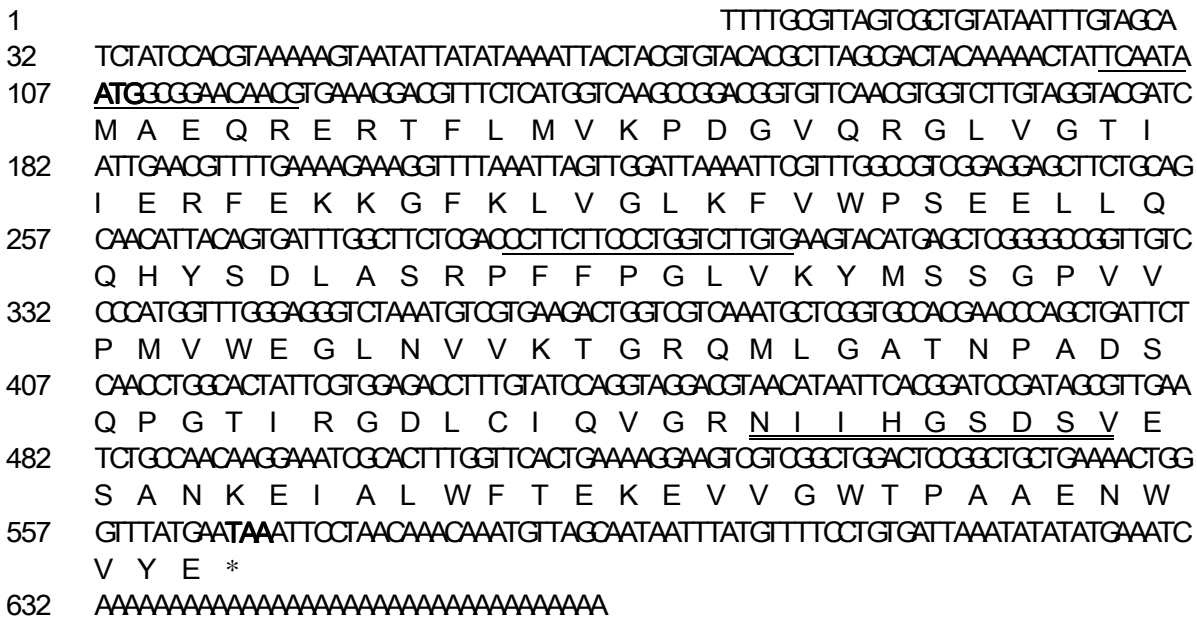


**Figure 1.** The complete nucleotide and deduced amino acid sequence of the *A. pernyi awd* gene. The amino acid residues are represented by one-letter symbols. The initiation codon ATG is indicated with bold and termination codon TAA is indicated with bold and by an asterisk. The nucleoside diphosphate kinases active site positioned at 116-124 (NIIHGSDSV) is double-underlined. The nucleotide underlined show the positions of gene specific primers used in the experiment.

ultraviolet spectrometer. Using 2 µg of total RNA per sample, first strand cDNA was generated with TIANScript cDNA Synthesize Kit (TIANGEN Biotech Co. Ltd., Beijing) following the manufacturer's instructions.

#### Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

The cDNA samples from the four developmental stages and various tissues were amplified by semi-quantitative RT-PCR method using the gene-specific primer pair LYQ122 (5' TCAAT AATGG CGGAA CAACG 3') and LYQ123 (5' CACAA GACCA GGGAA GAAGG 3') for the *awd* gene, which generated a 204 bp fragment. A fragment of 468 bp from the *actin* gene (GU073316; Wu et al., 2010) was amplified in parallel to each RNA sample using the primer pair LYQ85 (5' CCAAA GGCCA ACAGA GAGAA GA 3') and LYQ86 (5' CAAGA ATGAG GGCTG GAAGA GA 3'), as the internal control for adjustment of template RNA quantity. These primers were designed on the basis of the coding sequences. PCR amplification was performed in a total reaction volume of 25 µl, containing normalized cDNA, 20 pmol of each primer, 2 mM $MgCl_2$, 0.25 mM dNTP, 1 × buffer and 2.5 units of Taq DNA polymerase (TIANGEN Biotech Co. Ltd., Beijing). PCRs were performed with the following cycles: Initial denaturation at 95°C for 5 min followed by 25 cycles of 1 min at 95°C, 30 s annealing at 55°C, 30 s extension at 72°C; and a final extension at 72°C for 10 min. The amplification products were analyzed on 1.0% agarose gels stained with ethidium bromide. To avoid sample DNA contamination, the negative RT-PCRs control reactions were performed with total RNA as templates. The RT-PCR experiments were performed three times. To confirm the specificity of RT-PCR amplification, the RT-PCR products were purified from the gel and sequenced. Statistical analysis was performed with available statistical software SPSS v11.5. A two-tailed Student's test was used to determine the statistical difference between groups and $p < 0.01$ was considered as significant.

## RESULTS AND DISCUSSION

### cDNA cloning and sequence analysis of the *A. pernyi awd* gene

The *A. pernyi awd* gene was identified from a pupal *A. pernyi* cDNA library. The full-length cDNA sequence of the *awd* gene with the deduced amino acid sequence below the nucleotide sequence is shown in Figure 1. The isolated cDNA sequence was 666 bp in length, having a 5' untranslated region (UTR) of 106 bp, a 3' UTR of 63 bp with a poly (A) tail, and an ORF of 462 bp encoding a polypeptide of 153 amino acids. However, no polyadenylation signal sequence AATAAA was found. The G+C content of ORF of the *A. pernyi awd* gene was 48.05%. Predicted protein sequences of this cDNA shared 82% identity and 92% similarity with that of abnormal wing disks protein, also known as nucleoside diphosphate kinase, from *D. melanogaster* (GenBank accession no. CAA31500) (Biggs et al., 1988; Chiadmi et al., 1993). We therefore, referred to the protein as *A. pernyi awd* protein. This cDNA sequence has been deposited in GenBank under accession no. GU073317.

ScanProsite showed that the *A. pernyi awd* protein contained a nucleoside diphosphate kinases active site motif positioned at 116-124 (NIIHGSDSV) (Moréra et al., 1995; Lascu et al., 2000) and conserved multimer

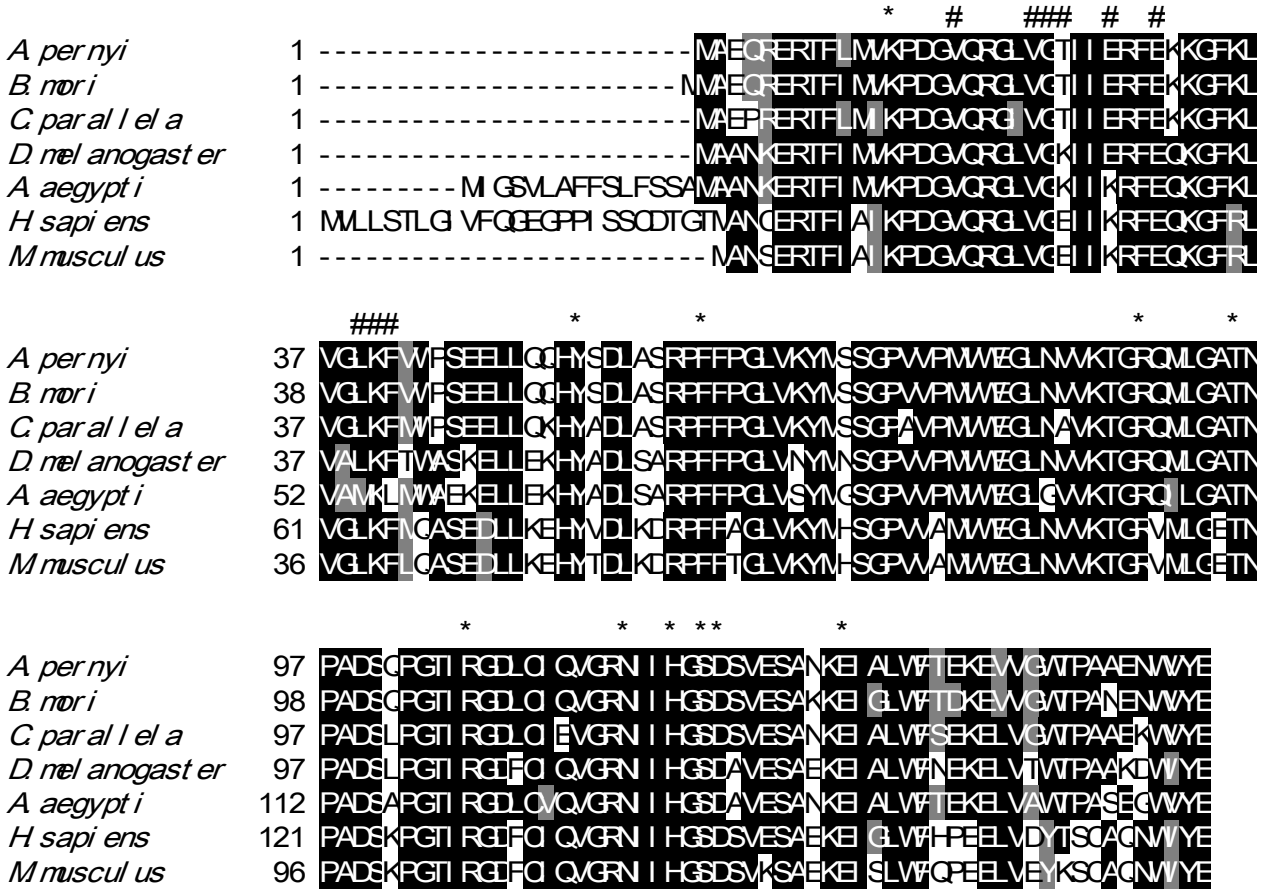


**Figure 2.** Sequence alignment of the *A. pernyi* awd proteins with its homologues. The awd proteins from *Bombyx mori* (ABF51506), *Choristoneura parallela* (AAM53644), *Drosophila melanogaster* (CAA31500), *Aedes aegypti* (XP_001662893), *Homo sapiens* (AAO85436), and *Mus musculus* (BAC28873) were included. The sign (*) shows the position of the nucleoside diphosphate kinases active site, and the sign (#) shows the position of multimer interface.

interface (Moréra et al., 1995; Lascu et al., 2000; Janin et al., 2000).

The predicted molecular weight of the *A. pernyi awd* protein was 17.15 kDa and isolectric point was 6.4. Protein signal peptide prediction revealed that no deduced signal peptide cleavage site in the N-terminal (Signal peptide probability: 0.000; Signal anchor probability: 0.000), meaning that it is a non-secretory protein. None of the transmembrane helices was detected in this protein by transmembrane protein topological structure analysis. Prediction of subcellular localization by SubLoc indicated it was a cytoplasmic protein (Reliability Index: RI = 3; Expected Accuracy = 84%). As these results were based on the predictive software, further experiments are needed to have additional evidences.

## Homologous alignment and phylogenetic analysis

To assess the relationship of the *A. pernyi awd* to *awds* from other organisms, identities were calculated based on a Clustal X alignment including 15 *awd* protein sequences (Figures 2 and 3). The deduced *A. pernyi awd* protein revealed 82 and 75% identity with that of *D. melanogaster* (CAA31500) and *Homo. sapiens* (AAO85436), respectively. The *A. pernyi awd* protein also revealed 91 and 96% identity with the putative *awd* protein from *Choristoneura parallela* (AAM53644) and *B. mori* (ABF51506), all of them belonging to Lepidoptera.

Sequence alignment revealed that these amino acid residues involved in the nucleoside diphosphate kinases active site (Moréra et al., 1995; Lascu et al., 2000) and multimer interface (Moréra et al., 1995; Lascu et al., 2000; Janin et al., 2000) are highly conserved in all the *awd* protein.

The phylogenetic tree constructed by MEGA version 4 (Tamura et al., 2007) using the NJ method (Saitou and Nei, 1987) is shown in Figure 3. All used *awd* sequences were branched into two groups in phylogenetic tree: Vertebrates and invertebrates (insects). Within insect group, the *awd* proteins from three lepidopteran moths assembled into one subgroup, and those from dipterans and two hemipterans into the other. In phylogenetic tree, the *A. pernyi awd* protein sequence was closely related to

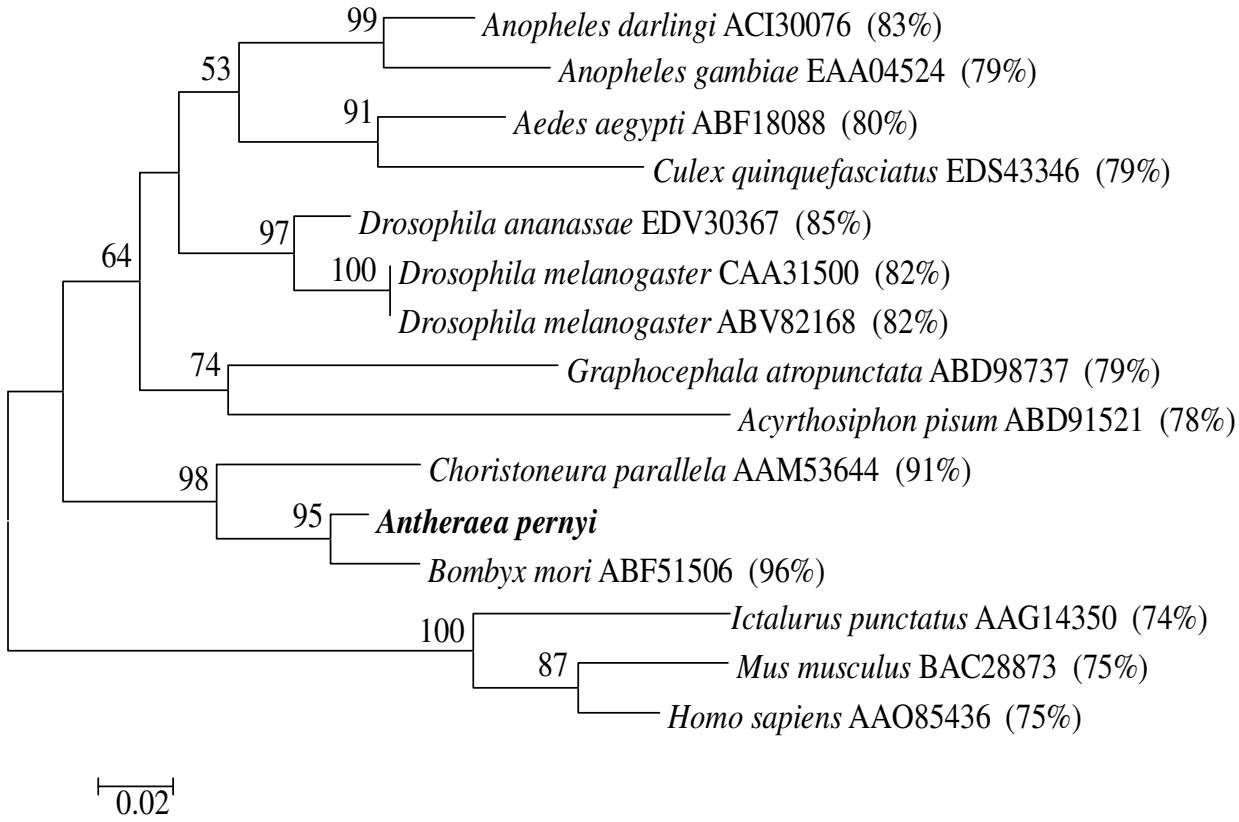


**Figure 3.** Phylogenetic tree based on the amino acid sequence comparisons of the *awd* proteins from various organisms. GenBank accession numbers of the *awd* protein are shown followed the names of organisms. Identities in parentheses are obtained by pairwise alignment of amino acid sequence of *A. pernyi awd* protein with indicated *awd* proteins.

that of *B. mori* with 95% confidence support.

## Expression patterns of the *awd* gene

Semi-quantitative RT-PCR was employed to detect and quantify the *A. pernyi awd* gene expression levels during different developmental stages and in tissue distributions in fifth-instar larvae. A constitutively expressed gene *actin*, which has been isolated in our laboratory (Wu et al., 2010), was used an internal control. Expression analyses of the *awd* gene during four developmental stages are shown in Figure 4. The results showed that the *awd* gene was expressed during four developmental stages. The expression level in the pupae was the highest, which was consistent with that observed in *B. mori* (Zhao et al., 2009). These results suggest that the *awd* gene products play an important role throughout the entire life cycle.

Tissue distributions of the *awd* gene during fifth instar larvae are shown in Figure 4. In this study, the *awd* RNA was present in all tested tissues including blood, midgut, silk glands, Malpighian tublues, spermaries, ovaries, brain, muscle, fat body, and body wall. The *awd* transcripts were most abundant in Malpighian tubules. The insect Malpighian tubules system is a type of excretory and osmoregulatory system, which consists of branching tubules extending from the alimentary canal that absorbs solutes, water, and wastes from the surrounding hemolymph (Beyenbach et al., 2010). The wastes then are released from the organism in the form of solid nitrogenous compounds. Therefore, the high expression of this gene in the Malpighian tubules corresponds to its role in the development of *A. pernyi*.

The *B. mori* is a major lepidopteran insect model for research. Large-scale EST resource and extensive microarray information for *B. mori* are available at GenBank, and SilkDB (Duan et al., 2009). The *in silico* gene expression analysis based on the EST sources and extensive microarray information revealed that the *B. mori awd* gene was also widely expressed, consistent with that observed in *A. pernyi*.

## Expression change of the *awd* gene under temperature stress

In order to examine the influence of temperature stress on *awd* expression, total RNA extracted from the fat body of *A. pernyi* pupae was used as template to detect the

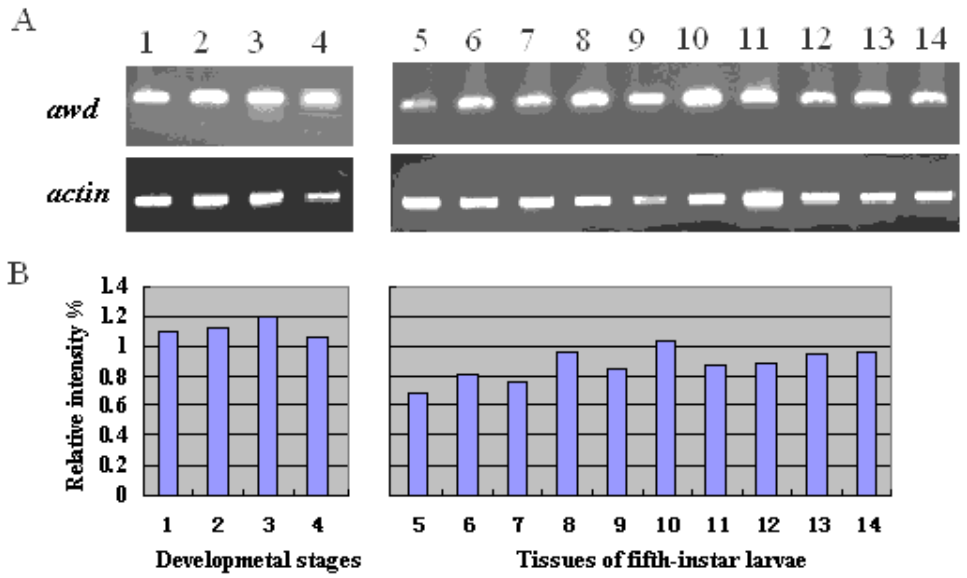


**Figure 4.** The expression patterns of the *A. pernyi awd* gene in different developmental stages and different tissues of the fifth-instar larvae. Both electrophoretic results (A) and the relatively intensity (B) are shown. The expression patterns of the a*wd* gene were analyzed by RT-PCR, which was amplified after 30 cycles with specific primer pairs for the *awd* or *actin* gene. Lanes: 1, Eggs at day 5; 2, moths; 3, pupae; 4, fifth instar larvae; 5, blood; 6, fat body; 7, midgut; 8, silk glands; 9, body wall; 10, Malpighian tublues; 11, spermaries; 12, ovaries; 13, brain; 14, muscle.

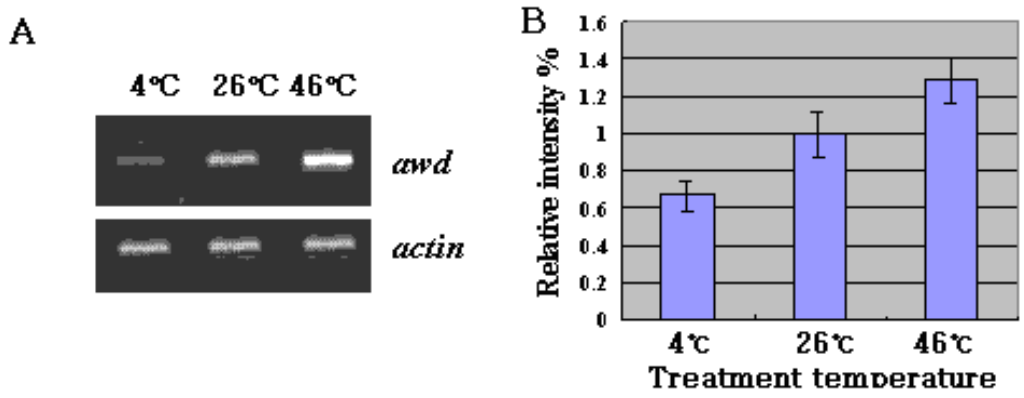


**Figure 5.** Semi-quantitative expression analysis of the *A. pernyi awd* gene in response of temperature stress including heat shock (46℃), control (26℃) and cold shock (4℃). Both electrophoretic results (A) and the relatively intensity (B) are shown. The mRNA expression level of the *awd* gene under 26℃ is defined as "1". The *actin* gene was used as control. A two-tailed Student's test was used to determine the statistical difference between groups and $P < 0.01$ was considered as significant.

levels of *awd* transcript after exposure to low- or high-temperature treatment by semi-quantitative RT-PCR analysis. The mRNA expression of the *A. pernyi awd* gene was significantly ($p < 0.01$) down-regulated after cold shock (4℃ for 3 h) and significantly ($p < 0.01$) up-regulated after heat shock (46℃ for 3 h) (Figure 5).

It has been known that the *Drosophila awd* protein is a microtubule-associated NDK, which plays a critical role in spindle microtubule polymerization (Biggs et al., 1990). Complete loss of function can cause lethality after the larval stage, and the imaginal discs during the larval stage are small and incapable of normal differentiation (Rosengard et al., 1989; Biggs et al., 1990). Loss of *D. melanogaster awd* protein results in dysregulated tracheal cell motility (Dammai et al., 2003). The *B. mori awd* gene has also been thought to be closely related to wing development (Zhao et al., 2009). In this study, the results indicated that the *A. pernyi awd* gene is inducible under temperature stress, and that its expression pattern is different after cold stress and heat stress.

In conclusion, we have cloned and characterized the 'abnormal wing disc protein' (*awd*) gene from *A. pernyi*, which is the third sequence of Lepidopteran insect. The study found that the *awd* gene is expressed during four developmental stages and in all tissues tested, suggesting that it plays a critical role in development of *A. pernyi*. The study also found that the *A. pernyi awd* gene may contribute to its temperature tolerance. It is hoped that the molecular characterization of *awd* protein in *A. pernyi* will expand the understanding of *awd* proteins.

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